



Protective effect of NAC against malathion-induced oxidative stress in freshly isolated rat hepatocytes

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ABSTRACT

Purpose: Induction of oxidative stress by Organophosphate compounds (OPs) has been previously reported. In the present work, the mechanism of protective effects of N-acetylcysteine as a glutathion (GSH) prodrug against malathion-induced cell toxicity was investigated. In this work, freshly isolated rat hepatocytes were used to determine the effect of NAC on malathion-induced cytotoxicity, formation of reactive oxygen species (ROS) and mitochondrial dysfunction. **Methods:** Rat hepatocytes were isolated using collagenase perfusion and then cell viability, mitochondrial membrane potential (MMP) and ROS formation were determined using trypan blue exclusion, Rhodamine 123 fluorescence and fluorogenic probe, 2', 7' -dichlorofluorescein diacetate (DCFH-DA), respectively. **Results:** Despite the protective effect of NAC on malathion-induced cell toxicity and MMP dysfunction, its efficacy against ROS formation was not adequate to completely protect the cells. **Conclusion:** Cytotoxic effects of malathion regardless of its cholinergic feature, is started with gradual free radical production but, the main factor that causes cell death, is mitochondrial dysfunction, so that reduction of ROS formation alone is not sufficient for cell survival, and the maintenance of mitochondrial integrity through different mechanisms is the most ameliorative factor specially at high levels of cell damage, as NAC seemed to protect cells with various fashions apart from ROS scavenging in concentrations higher than malathion's LC50.

Introduction

Malathion is one of the most widely used organophosphate compounds (OPs) applied in different fields like agriculture (as a pesticide), veterinary practice (as an ectoparasiticide), treatment of body lice on humans, food preparation and processing areas.¹ OPs are cholinesterase inhibiting chemicals and the main cause of pesticide poisonings.² Besides inhibiting cholinesterase (ChE), oxidative stress has been recently proposed as a main toxicity mechanism for OPs both in acute and chronic poisoning cases.³⁻⁵ Increased lipid peroxidation and diminished antioxidant defense capacity particularly glutathione are important findings accounted for OPs toxicity.⁶ Generation of oxidative intermediates induced by various agents has been predicated in cell death processes mainly mediated by the intracellular organelle mitochondria. Mitochondria⁷ are important intracellular source of ROS because of being the oxidative phosphorylation station in the cells and a susceptible target for damaging effects of ROS under certain pathological situations owing to abundance of tetraacyl phospholipid cardiolipin in their membrane.⁸⁻¹⁰ Mitochondrial membrane integrity is

important for efficient oxidative phosphorylation.¹¹ While increased ROS production is a harmful outcome of mitochondrial dysfunction¹², there is lots of evidence underlining the responsibility of mitochondrial impairment for ROS signaling via apoptotic pathways.¹³⁻¹⁴ Despite this, mitochondria have some antioxidant defense systems that help protect it against deleterious effects of ROS. The most important agents for mitochondrial antioxidant protection are the tripeptide glutathione (GSH) and multiple GSH-linked antioxidant enzymes.¹⁵

Considering oxidative stress induction by OPs pesticides, several studies proposed that a drug that could act as a multiplier of GSH content and a reductant would improve the cell tolerance towards OP-toxicity. In this regard, N-acetyl-L-cysteine (NAC) as a cell-permeable GSH prodrug that acts through direct scavenging of free radicals and revival of glutathione and cysteine can be a good candidate. Recently, our team reported that NAC can prevent toxicity of OPs in rats.¹⁶

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The liver that is of extraordinary importance in detoxification of toxic metabolites acts as a potential site for OPs- induced damages.^{17, 18} Since bio-transformation of thiono-OP as an imperative mechanism of their activation is mostly occurred in the liver, this organ is considered as one of the preliminary targets for malathion- induced toxicities.¹⁹

In this study, the role of mitochondrial dysfunction and enhanced ROS formation in malathion- induced cytotoxicity as well as the protective effect of NAC against it has been examined.

Materials and Methods

Chemicals

Bovine serum albumin, collagenase A from clostridium histolyticum and HEPES from Roche Diagnostics (Indianapolis, IN), 2',7'-dichlorofluorescein diacetate (DCFH-DA) and rhodamine 123 from Fluka (Italy), N-acetyl-cysteine (NAC) and 1-bromoheptane from Acros Pharmaceuticals, heparin sodium salt grade 1-A, trypan blue, methanol, and malathion 90% from Merck (Germany) were used in this study.

Animals

Male Sprague–Dawley rats (200–250 g) were obtained from the Laboratory of Animal Research Center of Tabriz University (Medical Sciences). The rats were housed in an air-conditioned room, under controlled temperature of $23 \pm 1^\circ\text{C}$, relative humidity of $36 \pm 6\%$ and 12 h light/12 h dark conditions for 1 week before starting the experiments. They were allowed to feed with standard laboratory chaw and tap water *ad libitum*. Procedures involving animals and their care were conducted in conformity with the NIH guidelines for the care and use of laboratory animals.

Preparation of hepatocytes

Hepatocytes were isolated from adult male Sprague–Dawley rats (220–250 g), by a two-step collagenase perfusion of the liver as described previously.²⁰ After isolation, the cells were suspended (10^6 cells/ml) in Krebs-Henseleit buffer (NaCl 118 mM; KCl 4.7 mM; KH_2PO_4 1.17 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.19 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.58 mM; NaHCO_3 25 mM) containing 12.5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and incubated under a stream of 95% O_2 and 5% CO_2 in continuously rotating round-bottomed 50 ml flasks at 37°C for 30 minutes before the addition of chemicals. Stock solutions of chemicals were made either in incubation buffer or in methanol and added to the hepatocyte suspensions at the indicated time points. Cell viability was measured by Trypan blue exclusion method.²⁰ The hepatocytes used in this study were at least 85-90% viable immediately after isolation.

Exposure of hepatocytes to malathion and NAC

According to a previous report on the toxicity and metabolism of malathion in isolated rat hepatocytes²¹

as well as our pilot studies, concentrations in the range of 0.5-1.5 mM were chosen to be used in this experimental set up. malathion was dissolved in methanol and NAC was dissolved in deionized double distilled water and added to the flasks containing the hepatocytes (10^6 cells /ml) at mentioned time intervals. 1- bromoheptane was dissolved in methanol and was added to the flasks 30 minutes before adding malathion. The utilized concentration of both NAC and 1- bromoheptane was 200 μM in cell suspensions.

Measurement of ROS formation

ROS formation was measured according to Eghbal et al., 2004b.²² Briefly, DCFH-DA was dissolved in methanol. The final concentration of DCFH-DA in cell suspensions (10^6 cells per ml) was 1 μM . DCFH-DA was added to the cell suspensions at the same time as malathion and a 100 μl aliquot was taken and diluted in 2ml of the incubation buffer. The samples were taken and the fluorescence intensity was measured at the excitation wavelength 485 nm and the emission wavelength 530 nm.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential in hepatocytes was assessed by monitoring the uptake of the cationic dye, rhodamine 123 as described.²³⁻²⁴ Isolated cells were extracted then resuspended in original media containing 1 μM rhodamine 123. After 10 minutes of incubation, the cells were centrifuged and the supernatant was measured with a Shimadzu RF-5000U spectrofluorimeter. The amount of dye remaining in the supernatant was inversely proportional to the membrane potential of the cells.

Statistical analysis

Statistical comparisons were carried out using a one-way analysis of variance (ANOVA) followed by the Bonferroni *t*-test (post-hoc) for multiple comparisons to determine statistical significance ($P < 0.05$) between treatments and control groups.

Results

Cytotoxic effect of malathion on freshly isolated rat hepatocytes

Figure 1 indicates the absolute toxicity of malathion in concentrations above 0.5 mM whereas the 1 and/or 1.5 mM caused significant cell death. Malathion at 2 mM caused 100% mortality 3 hours after incubation.

Effect of malathion on MMP in freshly isolated rat hepatocytes

Figure 2 illustrates the relative percentages of MMP in test groups in comparison with the control, where the 100 percent of Rhodamine 123 trapping occurred in the intact mitochondria. As shown, there was a significant difference in MMP of the malathion-treated cells (1 & 1.5 mM) in comparison with the control.

Effect of malathion on ROS formation in freshly isolated rat hepatocytes

As shown in figure 3, ROS formation was significantly increased in 1 and 1.5 mM of malathion-treated cells in comparison with the control while 0.5 mM did not show a significant effect.

Effect of 1-bromoheptane on malathion toxicity

Table 1 compares the different rates of malathion-induced cytotoxicity in the cells pre-incubated with 1-bromoheptane to that in the cells treated with different concentrations of malathion only. In the presence of 1-bromoheptane, the cytotoxicity of malathion was intensified and the rate of cell mortality was increased even with nontoxic concentrations of malathion (0.25, 0.5 mM).

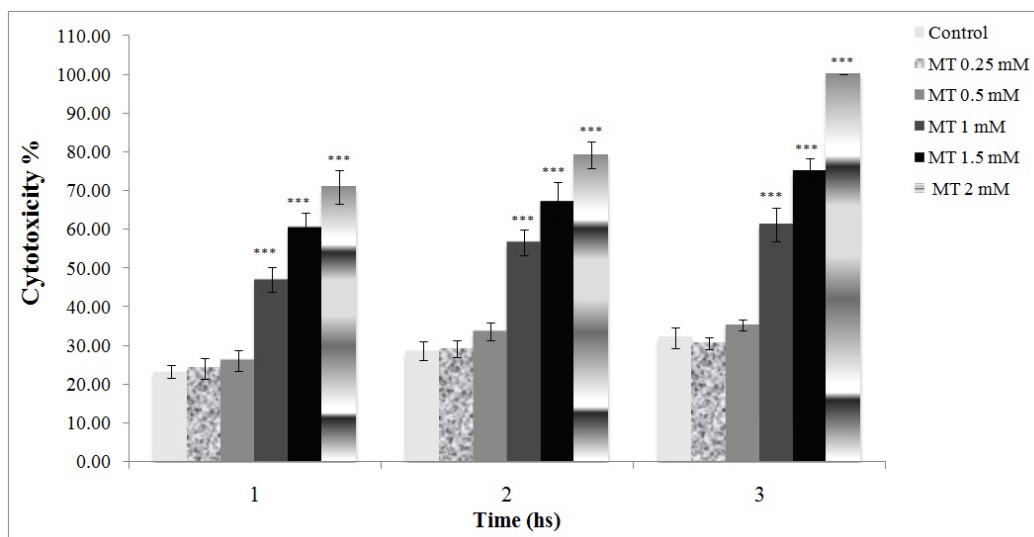


Figure 1. Cytotoxic effect of malathion on freshly isolated rat hepatocytes

Malathion was added to the flasks containing the hepatocytes to make the concentrations (1 and 1.5 mM) and cell viability was measured with uptake of trypan blue. All results were shown in mean \pm S.E.M and are at least from 3 independent experiments. MT (malathion)

***Shows significant difference ($p < 0.001$) with control

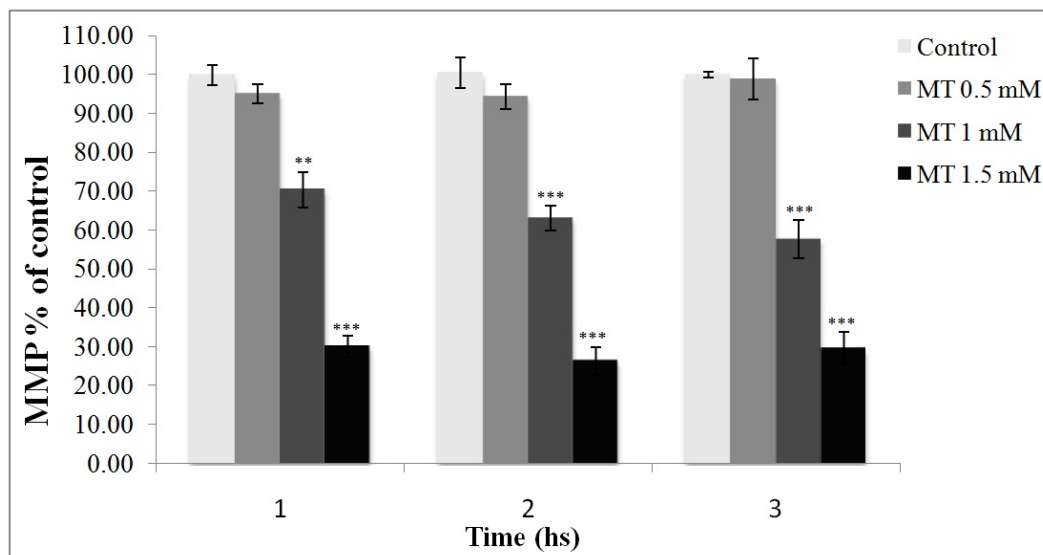


Figure 2. Effect of malathion on mitochondrial membrane potential in freshly isolated rat hepatocytes

Rhodamine was added to the aliquots of cell suspensions and the fluorescence amounts of the samples were measured with a Shimadzu RF-5000U spectrofluorimeter. The amount of dye remaining in the supernatant was inversely proportional to the membrane potential of the cells. All results were shown in mean \pm S.E.M and are at least from 3 independent experiments. MT (malathion), MMP (mitochondrial membrane potential)

**Shows significant difference ($p < 0.01$) with control

***Shows significant difference ($p < 0.001$) with control

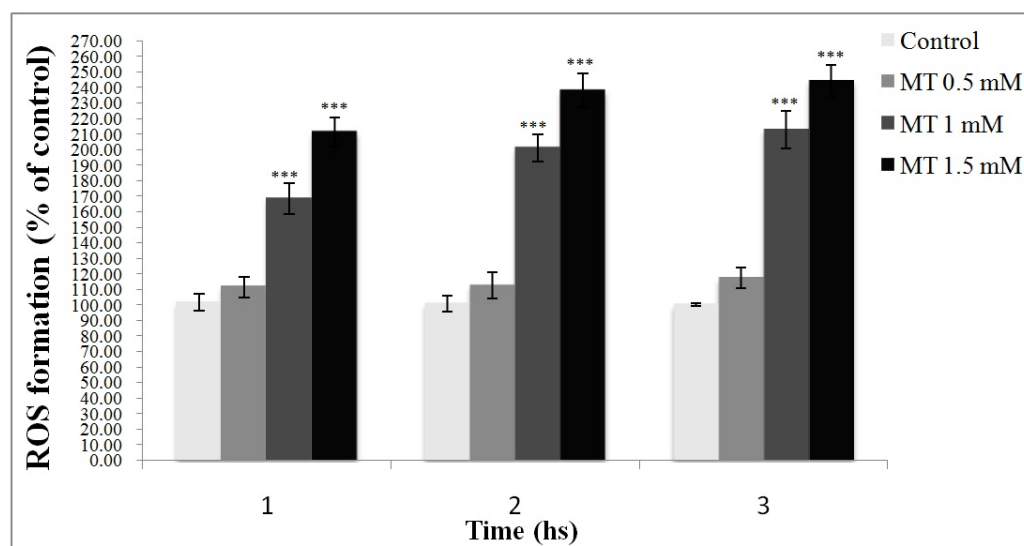


Figure 3. Effect of malathion on ROS formation in freshly isolated rat hepatocytes

DCF was added to the flasks containing hepatocytes at the same time as malathion and the fluorescence intensity was measured at the excitation wavelength 485 nm and the emission wavelength at 530 nm. All results were shown in mean \pm S.E.M and are at least from 3 independent experiments. MT (malathion), ROS (reactive oxygen species)

***Shows significant difference ($p < 0.001$) with control

Table 1. Effect of 1-bromoheptane on malathion cytotoxicity

Treatments	Time Intervals		
	Cytotoxicity (%)		
	1 Hs	2 Hs	3Hs
Normal Cells (without treatment)	23 \pm 1.7	28 \pm 2.3	32 \pm 2.7
“ + MT 0.25mM	24 \pm 2.7	29 \pm 2.1	30 \pm 1.4
“ + MT 0.5mM	26 \pm 2.6	33 \pm 2.3	35 \pm 1.4
“ + MT 1mM	47 \pm 3.2 ^c	56 \pm 3.3 ^c	61 \pm 4.4 ^c
“ + MT 1.5mM	60 \pm 3.8 ^c	67 \pm 5.2 ^c	75 \pm 3.1 ^c
1-BH 200 μ M treated Cells	24 \pm 3.1	31 \pm 3.4	32 \pm 2.3
“ + MT 0.25mM	43 \pm 2.8 ^b	46 \pm 3.4 ^a	49 \pm 3.5 ^a
“ + MT 0.5mM	48 \pm 4.1 ^b	55 \pm 2.9 ^b	57 \pm 4.3 ^b
“ + MT 1mM	54 \pm 2.3 ^c	81 \pm 2.6 ^c	83 \pm 4.6 ^c
“ + MT 1.5mM	72 \pm 3.4 ^c	86 \pm 3.2 ^c	100 \pm 0.0 ^c

Hepatocytes were incubated in Krebs–Henseleit solution, pH 7.4 at 37 °C under the atmosphere of 95%O₂/5%CO₂. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion method. All data were shown in mean \pm S.E.M and are at least from 3 independent experiments.

a: Significant difference ($p < 0.05$) with control

b: Significant difference ($p < 0.01$) with control

c: Significant difference ($p < 0.001$) with control

Protective effect of NAC against malathion-induced cytotoxicity

Figure 4 shows that NAC protected hepatocytes against malathion-induced cytotoxicity at different time intervals.

Protection by NAC of MMP against malathion toxicity

As shown in figure 5, NAC was able to prevent the reduction of MMP in malathion-treated cells in all 3 time intervals.

Effect of NAC on malathion-induced ROS formation

As shown in figure 6, NAC was able to prevent malathion- induced ROS formation only in the cells treated with 1 mM malathion.

Discussion

This study evaluated the cytotoxic effects of malathion as well as the protective effect of NAC against it in freshly isolated rat hepatocytes. The results indicate that NAC has beneficial effects on some parameters of malathion- induced cytotoxicity so that the decrease in

cell mortality, modification of altered MMP and reduction of ROS formation are obvious. The results of the present study indicate that hepatocytes were able to tolerate malathion in concentrations less than 0.5 mM, while the cell viability was significantly decreased at

higher concentrations so that 100% cell mortality was seen in the group receiving 2 mM malathion after 3 hours incubation and the LC50 was estimated to be around 1mM in this experimental set-up.

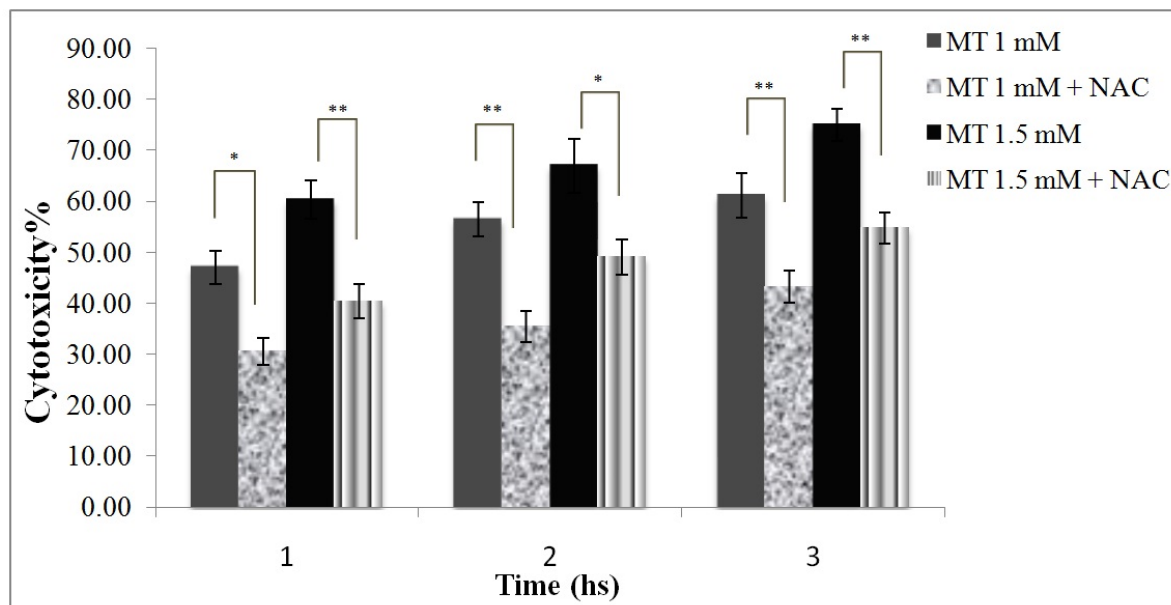


Figure 4. Protective effect of NAC against malathion toxicity in freshly isolated rat hepatocytes
Malathion (1 and 1.5 mM) and NAC (200μM) were added to the flasks containing the hepatocytes and cell viability was measured with uptake of trypan blue. All results were shown in mean \pm S.E.M and are at least from 3 independent experiments. MT (malathion), NAC (N-acetylcysteine)
* Shows significant difference ($p < 0.05$)
**Shows significant difference ($p < 0.01$)

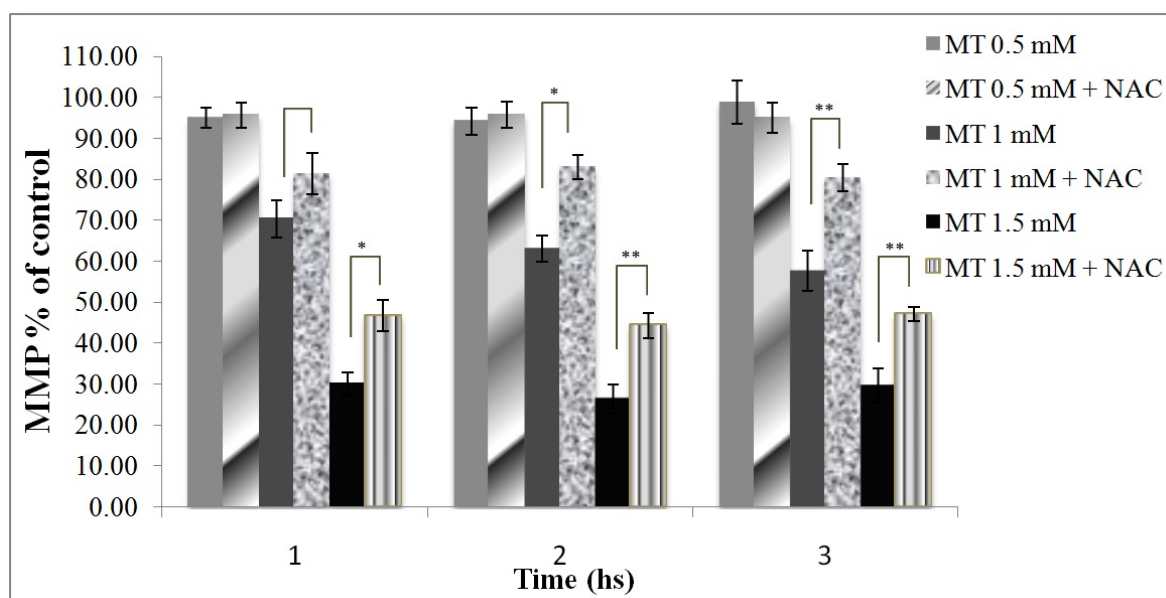


Figure 5. Protection of mitochondrial membrane potential against malathion toxicity by NAC
Rhodamine was added to the aliquots of cell suspensions and the fluorescence amounts of the samples were measured with a Shimadzu RF-5000U spectrofluorimeter. The amount of dye remaining in the supernatant was inversely proportional to the membrane potential of the cells. All results were shown in mean \pm S.E.M and are at least from 3 independent experiments. MT (malathion), NAC (N-acetylcysteine), MMP (mitochondrial membrane potential)
* Shows significant difference ($p < 0.05$)
**Shows significant difference ($p < 0.01$)

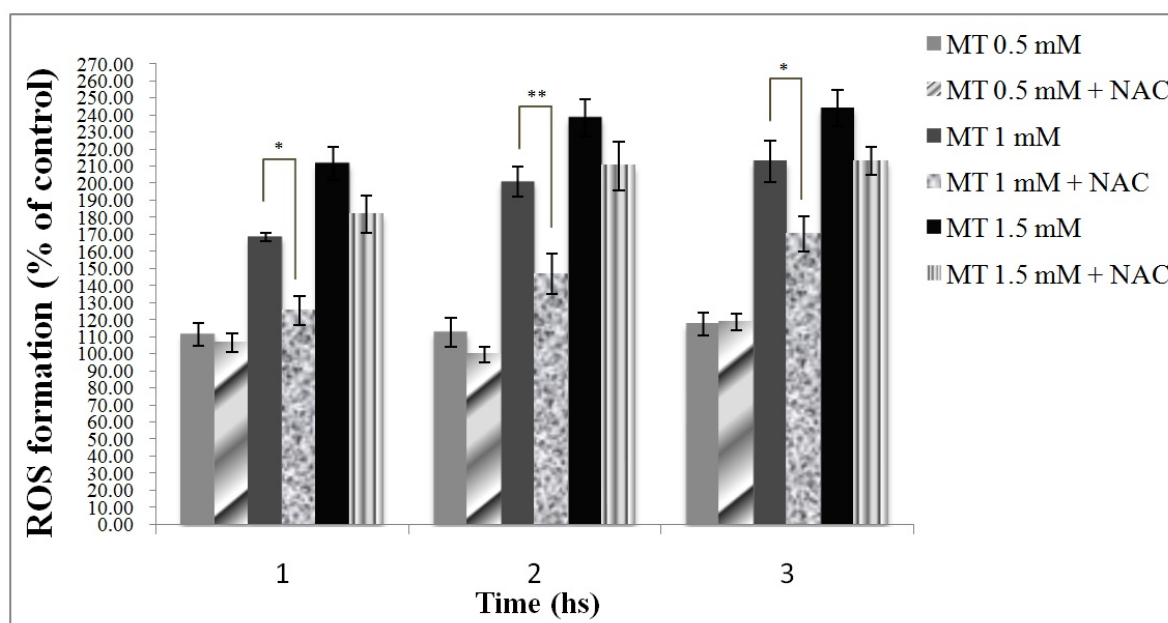


Figure 6. Effect of NAC on malathion – induced ROS formation in freshly isolated rat hepatocytes

DCF was added to the flasks containing hepatocytes at the same time as Malathion and the fluorescence intensity was measured at the excitation wavelength 485 nm and the emission wavelength at 530 nm. All results were shown in mean \pm S.E.M and are at least from 3 independent experiments. MT (malathion), NAC (N-acetylcysteine), ROS (reactive oxygen species)

* Shows significant difference ($p < 0.05$)

**Shows significant difference ($p < 0.01$)

Malathion in its toxic concentrations in isolated rat hepatocytes (1, 1.5 mM) significantly increased ROS formation which is in parallel with the result of the previous studies showing the oxidative effects of various OPs in vitro²⁴, and also in vivo.²⁶⁻²⁸

The basis of OP toxicity in induction of oxidative stress has been speculated in two prospects; (a) redox-cycling activity recounted as the ability to accept an electron and produce free radicals that is subsequently followed by superoxide anions and hydrogen peroxide formation through transmission of an electron to oxygen and dismutation reactions, respectively; and (b) antioxidant depletion because of disruption of antioxidant homeostasis that results in increased ROS formation.²⁹⁻³²

This study not only confirmed the previous reports on the prooxidative activity of OPs, but also examined if the toxicity of malathion could be an outcome of mitochondrial permeability transition (MPT). According to the results obtained from the rhodamine 123 uptake experiment, the loss of MMP significantly occurred in isolated rat hepatocytes incubated with 1 and/or 1.5 mM malathion. MPT is an important mechanism in various hepatotoxicities and defined as an abrupt increase in the inner mitochondrial membrane permeability to solutes of size less than 1500 Da. As a lethal event for the cells, MPT occurs with uncontrolled proton influx into matrix and is accompanied with dissipation of MMP, syncope of ATP synthesis, efflux of intramitochondrial ions specially calcium as well as mitochondrial swelling

because of osmotic influx of water. It can be mediated by oxidative stress and causes increased oxidative stress.^{24,33}

Two main functions of mitochondria are maintenance of cellular energy metabolism and regulation of cell death showing the unrivaled capacity of this organelle in ROS production. Indeed, mitochondrial respiratory chain is not only the main source of ROS production but also a sensitive target for the harmful effects of ROS. Ranjbar et al (2010)³⁴ have previously reported that malathion-induced mitochondrial dysfunction in rat brain is mostly mediated through oxidative stress. Since, release of cytochrome *c* toward triggering apoptosis can be largely mediated by mitochondria-generated ROS³⁵ as well as close correlation between apoptosis and collapse of MMP.³⁶ It can be ideated that hepatotoxic effect of malathion is mediated through occurrence of MPT toward programmed cell death. In addition, mitochondrial antioxidant defense system has a crucial role in the survival of cells.³⁷ But the most important factors for mitochondrial antioxidant protection are the tripeptide GSH and multiple GSH-linked antioxidant enzymes.^{38,39} So we evaluated the effect of GSH-depleted condition induced by 1-bromoheptane on malathion cytotoxicity. 1-Bromoheptane and other 1-Halogenoalkanes are GSH-S-transferase substrates and produce a substantial depletion of cellular GSH with minimal effects on phase I biotransformation and minimal cytotoxicity. 1-bromoheptane is the most effective and least cytotoxic GSH-S-transferase substrate tested, and therefore has

been used as a tool to modulate cellular GSH levels in isolated hepatocytes.⁴⁰ It is also clear that GSH depleted cells have adequate antioxidant and enzymatic systems to counteract physiological oxidative stress. It has been reported that treatment of cells with 1-bromoheptane depleted GSH by 87% within 30 min and the cells remained GSH-depleted for 4 hrs without significant cytotoxicity. A 30 fold higher concentration of 1-bromoheptane was required before cytotoxicity ensued. Therefore, 1-bromoheptane is particularly useful for studying the role of GSH in modulating xenobiotic cytotoxicity.^{40,41} Paying attention to noticeable increase in mortality of hepatocytes incubated with 1-bromoheptane in this study, GSH depletion is considered as a main mechanism of malathion-induced toxicity. In other words, GSH plays a critical role in detoxification of malathion. As shown, in the presence of 1-bromoheptane, cytotoxicity of malathion was intensified and the rate of cell mortality was increased even with nontoxic concentrations of malathion (0.25, 0.5 mM)(table 1).

NAC, as a precursor of the sulfur amino acid, cysteine, exerts antioxidative effect by reacting directly with electrophiles or by facilitating generation of GSH, after incorporation into cells. It is a small molecule that passes freely through cell membranes into the intracellular compartments and is subsequently deacylated to deliver cysteine. As an antioxidant, NAC directly scavenges hydrogen peroxide, hydroxyl free radicals and hypochloric acid *in vitro*.⁴²⁻⁴⁴ Furthermore, Saito et al⁴⁵ showed that administration of NAC or GSH results in a rapid and complete recovery of hepatic GSH level within 1 hour in mice and that the efficacy in supplying cysteine for recovery of GSH level and ROS scavenging in liver was similar between NAC and GSH treatment.

In this experimental set-up, NAC decreased malathion-induced cytotoxicity and ROS formation. Likewise, malathion-induced loss of MMP in freshly isolated rat hepatocytes was markedly terminated following NAC treatment. But the point is that NAC in malathion 1 mM-treated group declined cytotoxicity in parallel with both reduction of ROS formation and MMP obviation, whereas its cytoprotective effect in the next group (malathion 1.5 mM) is more similar to the pattern of MMP than the ROS one (Figures 5 & 6). Hence, it seems that maintenance of the cell survival is more related to the mitochondrial function integrity than the cellular ROS level, as the recent works on cell death mechanisms has focused on MPT. The mitochondrion has two redox-sensitive sites; one of them under the effect of mitochondrial GSH and the other influenced by NAD(P)H that can be modulated by ROS.⁴⁶ So, depletion of mitochondrial GSH may be considerably responsible for sensitization of cells to oxidative injury.⁴⁷ In hepatocytes, mitochondria include ~ 10-15 % of the total cellular GSH content. Lack of the enzymatic machinery for de novo GSH synthesis in mitochondria makes it dependent on GSH uptake from the cytosol by

carrier-mediated transport systems located in the inner membrane. It means that inconvenience of the mitochondrial membrane can violate the function of these carriers towards the mitochondrial dysfunction.⁴⁸⁻⁵⁰ NAC reduced toxicity in malathion 1.5 mM-treated hepatocytes in parallel with MMP upgrade but not distinctly ROS reduction. Thus, other mechanisms in addition to the ROS scavenging are responsible for the mitochondrial protective effects of NAC. In this regard, improvement of cellular energy supply is one of these proposed possible mechanisms, as the increasing effect of NAC on cytochrome c oxidase activity in mice synaptic mitochondria has been reported by Banaclocha & Martinez (1999).⁵¹ Furthermore, Saito et al (2010)⁴⁵ explained that ameliorative effect of NAC on cellular ATP level in the liver may be the result of its capacity to supply substrates needed for Krebs cycle. Thereupon, improvement of energy supply to mitochondria may be responsible for the accelerated uptake of cytosolic GSH into mitochondria as was observed after GSH treatment previously.⁴⁵

Conclusion

Based on the results of the current study, we could conclude that the cytotoxic effects of malathion as a common OP, regardless of its cholinergic effect, is started with gradual free radical production but, the main factor that causes cell death, is mitochondrial dysfunction, so that reduction of ROS formation alone is not sufficient for cell survival, and the maintenance of mitochondrial integrity through different mechanisms is the most ameliorative factor specially at high levels of cell damage, as NAC seemed to protect cells with various fashions apart from ROS scavenging in concentrations higher than malathion's LC50.

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Conflict of interest

Authors declare no conflict of interest.

Abbreviations

OPs (Organophosphate Compounds), ROS (Reactive Oxygen Species), MMP (Mitochondrial Membrane Potential), GSH (Glutathione), NAC (N-Acetylcysteine), DCFH-DA (2', 7' -Dichlorofluorescein Diacetate), LC50 (Lethal Concentration 50)

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